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CpG island methylation is an epigenetic modification of DNA associated with the silencing of gene transcription. The hypothesis of this proposal is that breast cancers develop along different pathways, some involving aberrant CpG island methylation for gene inactivation. Some of the genes or CpG islands identified in the methylation-dependent pathways will also be inactivated early in breast cancer evolution and/or will be associated with prognosis. These hypotheses will be tested in innovative studies that will identify novel CpG islands methylated in stage I or III breast cancers using the new technique of methylation specific representation differential analysis. The frequency and timing for methylation of these novel CpG islands will be defined in a case control study of ductal cell carcinoma *in situ*, stage I, and stage III ductal cell carcinomas. This study is collecting data on other risk factors (e.g., hormone profiles, alcohol intake) for breast cancer. In addition, cellular and genetic endpoints that are potentially prognostic for this disease: cell proliferation index (Ki-67), HER2/neu, progesterone receptor and estrogen receptor expression are being determined in this ongoing study. The novel technique of methylation-specific polymersae chain reaction will be used to detect methylated alleles in DNA from fixed tissue. Together, these results will identify novel CpG islands in breast cancer, define their timing for inactivation, identify pathways that may lead to breast cancer through gene inactivation by methylation, and identify markers of prognosis.

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(4) Introduction

The development of breast cancer may involve the inactivation of many different genes potentially resulting in several different "pathways" along which breast cancers evolve. Therapeutic approaches which target these pathways could result in improved survival rates and potentially cures for early stage disease. The goal of this study is to identify novel genes involved in breast cancer and to determine whether pathways exist that target these genes for inactivation. This study will focus on one specific mechanism by which genes are inactivated, the addition of a methyl group to cytosines within the promoter region of the gene, a phenomenon termed CpG island methylation. Aberrant CpG island methylation blocks the expression of the gene, thereby causing it to lose its normal function. Several genes already identified, p16, HIC1, estrogen, and E-cadherin are altered by this mechanism in breast cancer. The studies proposed will be focused on the identification of novel genes which are inactivated in breast cancer through CpG island methylation. The timing for inactivation and whether inactivation is associated with prognosis will also be determined. Novel genes inactivated by CpG island methylation will be identified by a PCR-based technique called methylation specific representational differential analysis (1). The novel PCR fragments will be sequenced and a homology search of GenBank using the BLAST program will be conducted. The methylation state of these novel CpG sequences will then be determined in tumor and normal pairs (n = 15) of both stage I and III ductal carcinoma. If coding sequence can be identified, RNase protection assays will also be conducted on tumor and normal pairs or breast cancer-derived cell lines to confirm that methylation within the CpG island leads to loss of expression. Finally, the frequency and timing for methylation of the novel CpG island containing genes will be defined in a case-control study. A New Mexico Women's Health Study is being conducted within the Epidemiology and Cancer Control Program at the University of New Mexico. This case-control study is enrolling 500 cases (300 non-Hispanic whites; 200 Hispanic whites) throughout New Mexico and a subset of these samples will be evaluated. Persons eligible for study participation

will have a primary diagnosis of carcinoma of the breast (in situ and Stages I, II, and IIIA). This study is investigating potentially modifiable prognostic factors for breast cancer such as hormone status, dietary and alcohol intake, and reproductive history. In addition, cellular and genetic endpoints that are potentially prognostic for this disease: cell proliferation index (Ki-67), HER2/neu, progesterone receptor and estrogen receptor expression are being determined in this ongoing study. Together, these results will identify novel CpG islands in breast cancer, define their timing for inactivation, identify pathways that may lead to breast cancer through gene inactivation by methylation, and identify markers of prognosis.

(5) Body

A brief overview of the new strategy for novel gene identification that was discussed in detail in the progress report for year 1 is provided. In the original application, we proposed to use the technique of methylation sensitive restriction fingerprinting developed by Gonzalgo et al. (2) and Huang et al. (3). However, in the interim period between submission of our proposal and funding, our major collaborator's laboratory (Dr. Stephen Baylin, Johns Hopkins) developed another technique (1) that appeared more robust for identifying differentially methylated CpG islands in cancer. Therefore, we elected to apply this technique to the identification of methylated CpG islands in breast cancer.

Near the end of year one we had generated plasmid preparations were from 192 colonies from the moderately GC rich subtraction library and the insert was amplified by PCR using T7 and T3 sequences surrounding the insert. All colonies selected contained inserts that varied in size from approximately 200 - 800 basepairs. Because Alu sequences are often methylated in normal tissue, it is important to first screen the colonies for this repetitive sequence. The PCR products from the 192 colonies were spotted onto nylon membranes and hybridized with a ^{32}P-labeled human Alu repetitive sequence. Sixty of the 192 inserts hybridized to the repetitive Alu sequence and were eliminated from further screening approaches.

Ten inserts from the moderately GC rich substraction library were further characterized. First, DNA from three breast cancer-derived cell lines, MCF-7 (the tester), MDBA-231, and MDBA-435, and the pooled normal DNA (driver) were spotted onto nylon membranes. All of these DNA samples were first subjected to the digestion protocol, ligation with the two different adaptors, and PCR amplification to generate the putative methylation specific amplicons. Each of the inserts was labeled with ^{32}P and hybridization to the 3 cell lines and normal tissue was assessed. Five of the inserts hybridized to all breast cancer cell lines and normal tissue indicating a lack of differential methylation. Two of the inserts hybridized selectively only to MCF-7, while three of the inserts hybridized to all 3 of the breast cancer-derived cell lines, but not the normal tissue. These 5 inserts were then subjected to sequencing. The size of these inserts ranged from 370 - 580 base pairs with GC contents of 48 - 60% (indicative of CpG islands).

Database searches were conducted for all 5 of the sequenced inserts and all matched sequence data that had been deposited as part of the human genome project. One of the inserts appears to be in the promoter region of a known gene not previously described in cancer, but proposed to function in the transport and uptake of cholesterol. Using the Gene Scan Analysis Software (MIT, Boston, MA), one of the clones appears to be within a GC region located near a putative promoter that lies upstream of an open reading frame that codes for a predicted protein of approximately 1900 amino acids. At the beginning of year 2, we began further characterization of all 5 inserts that included determining the methylation-state of these 5 CpG islands in tumor normal primary breast samples. Unfortunately none of these inserts proved to be methylated in primary tumors.

At this point, we elected to switch from the moderately GC rich substraction library to the other GC rich (adapters that should amplify regions that are approximately 70% GC rich) subtraction library that we had generated during year 1. Plasmid preparations were prepared as described above for 100 clones that were ultimately sequenced. Fifty unique sequences were

discovered from these 100 clones. Only two of the 50 clones did not match known sequences in the GenBank database. The size of these inserts ranged from 245 - 580 base pairs with GC content of 56 - 76% (indicative of dense CpG islands). In addition, 20 clones were homologous to CpG islands or 5' upstream regions of known genes. Based on whether the clones identified a known gene or putative gene we selected 9 clones to further characterize for methylation in breast cancer cell lines.

The first clone characterized was the 5' region of the BAK1 gene. The BAK1 gene functions as an antagonist of the BCL-2 gene in the apoptosis. The examination of 10 breast cancer cell lines that included MCF-7 (the cell line used in the substraction) failed to detect any evidence of methylation of this 5' region. Therefore, further studies were not conducted on this clone.

The second clone characterized contained a portion of the promoter region for the transcription factor PAX 5. The PAX genes encode nuclear transcription factors implicated in the control of mammalian development. The PAX5 protein has been shown to bind to a sequence in the 5' region of the p53 gene and inhibit p53 expression (4). Further examination of the genomic structure of the PAX5 genomic region revealed that two different proteins are derived from this gene. Similar to the p16p19 genes, the PAX5 locus contains two promoters, exon 1 α and exon 1 β, and 9 shared exons (5). Our subtraction library had contained the 5' sequence to exon 1 β. Because a CpG island was also located in the promoter for exon 1 α we decided to examine both promoters for methylation in breast cancer cell lines. Both the α and β PAX5 genes were methylated in cell lines with prevalences of 90% (9/10 cell lines) and 60% (6/10), respectively. Cell lines in which the PAX5 α and β genes were methylated also showed no transcript based on RT-PCR indicating that the gene was functionally inactivated. Furthermore, treatment of cell lines with the demethylating agent 5-deoxyazacytidine restored expression of the PAX5 α and β genes. We developed primers to conduct methylation specific PCR (MSP) on primary breast tumors. Methylation of the PAX5 α and β genes was seen in 21

of 30 and 15 of 30 tumors, respectively. We are currently bisulfite sequencing the promoter areas assayed by MSP in a subset of primary tumors and cell lines to determine the density of methylation within the α and β promoter regions. Thus, we have identified two genes that encode nuclear transcription factors that are frequently inactivated in breast cancer. A manuscript is in preparation to detail these findings.

Our statement of work for year 2 indicated that we should have identified genes inactivated by methylation, determined their prevalence in cell lines and primary tumors, verified that the methylation detected was associated with loss of transcription, and prepared a manuscript detailing the findings. All of these goals essentially have been accomplished. The goals for year 3 were to expand our analysis of the novel methylated genes to determine their timing for inactivation in a large collection of ductal carcinoma *in situ* lesions and ductal carcinomas, stage I to III. Due to the exciting findings with the PAX5 genes, we are electing to first screen the other 7 interesting clones. Depending on our findings, i.e. whether the clones prove to be methylated in the cell lines, we would first elect to characterize the new genes and if time permits begin looking at timing for inactivation.

(6) Key Research Accomplishments

Sequenced 100 clones from the GC-rich differential analysis subtraction library.

Identified 50 clones that contained unique sequences.

Identified 20 clones located in CpG islands or 5' upstream regions of known genes.

Initiated characterization of 9 clones.

Found that the BAK1 gene contained in one of the clones was a false positive and not methylated in breast cancer-derived cell lines.

Identifed the PAX5 α and β genes as being methylated in breast cancer derived cell lines.

Verified that methylation of both the PAX5 α and β genes was associated with gene silencing.

Demonstrated that methylation of the PAX5 α and β genes is a common event in primary breast tumors.

(7) Reportable Outcomes

None to date.

(8) Conclusions

During the second year of this study, we successfully identified two genes, PAX5 α and β that are commonly inactivated in primary breast tumors by promoter hypermethylation.

(9) References

1. Toyota, M., Ho, C., Ahuja, N., Jair, K-W, Li, Q., Toyota, M., Baylin, S.B., and Issa, J-P J. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res. 59: 2307-2312, 1999.

2. Gonzalgo, M.L., Liang, G., Spruck, C.H., Zingg, J-M, Rideout, W.M., and Jones, P.A. Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. Cancer Res. 57: 594-599, 1997.

3. Huang, T.H., Laux, D.E., Hamlin, B.C., Tran, P. Tran, H., and Lubahn, D.B. Identification of DNA methylation markers for human breast carcinomas using the methylation-sensitive restriction fingerprinting technique. Cancer Res. 57: 1030-1034, 1997.

4. Stuart, E.T., Haffner, R., Oren, M., and Gruss, P. Loss of p53 function through PAX-mediated transcriptional repression. EMBO Journal 14: 5638-5645, 1995.

5. Busslinger, M., Klix, N., Pfeffer, P., Graninger, P.G., and Kozmik, Z. Deregulation of PAX-5 by translocation of the Eu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. Proc. Natl. Acad. Sci. USA 93: 6129-6134, 1996.

(10) Appendix

None attached